5. Title: Study of Intestinal Immunoglobulins

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Objective The purpose of this investigation is to determine the type and amounts of immunoglobulin proteins found in human small intestinal fluid. Originally it was planned to also determine if these immunoglobulins have antibody activity, but to date studies have been only on specimens from normal patients, as technical problems in studying these protiens first had to be overcome.

Progress The past year has seen considerable progress in understanding what had originally been confusing findings as regards the gut juice immunoglobulins. Methods have been developed for dealing with technical problems as they arose. These will be described in detail.

1. Before any meaningful immunologic investigations of intestinal fluid can be undertaken, proteolytic enzyme activity has to be greatly suppressed or abolished. In fact, one in vitro method used in basic immunochemistry involves peptic and tryptic digestion of intact immunoglobulin molecules. This results in the elaboration of the subunits of immunoglobulins, the so-called H and L chains, which in various combinations represent the Fab, Fc and other functional fragments of immune globulins. Attempts by others to prevent proteolytic activity against immunoglobulins in intestinal juices under study involved the use of specific inhibitors, buffers with pH ranges inhibitory to enzymes, and attempts to remove heavy metal cofactors from the secretions by a variety of techniques. None of these have proven useful, since they do not inhibit all the enzymes which are present in gut secretions, and immunoglobulin breakdown continues.

Within the past year a method has been developed in the course of our work which apparently has solved this problem. It involves simply heating the secretions at 56°C for 30 minutes. After so doing, immunoglobulins are apparently unaltered significantly, and proteolysis does not occur against them. This has been shown to be the case by the following experiment: Two aliquots of the same intestinal juice were thawed. One was heated at 56° for 30 minutes; the other was not. An equal portion of normal human serum was then added to each specimen, and both were incubated at 37° for 24 hours. At zero time and at the first, third, seventh, fifteenth, and twenty-fourth hours a specimen of each was obtained and studied by immunoelectrophoresis. A portion was also placed on agar plates with incorporated specific antisera for quantitation of immunoglobulins (see below). In the intestinal juice not previously heated, immunoglobulins IgA, IgG and IgM were swiftly destroyed; at zero time some destruction of all three was already apparent*, and it was complete by three hours in the case of IgG, and by seven hours for IgA, and IgM. Quantities of all three classes of immunoglobulins fell sharply by one hour, and thereafter only indistinct precipitates appeared in the agar diffusion plate. In the heated intestinal juice, immunoelectrophoresis of samples showed no apparent alteration or loss of the three classes of immunoglobulins, and their quantity fell no more than 10% after 24 hours of incubation at 37 C. This experiment, which con-

*presumably digestion took place during the course of immunoelectrophoresis, probably in the antigen well after the specimen was added.

firms the ability of heating to markedly impair the capacity of juice to destroy added immunoglobulins, is readily reproducible, By analogy, it was therefore expected that those immunoglobulins normally present in the secretions would likewise by protected by preliminary heating. This has been born out in practice, and now all juices studied are so heated as the initial step in their processing.

Fluids are now collected from normal subjects by intestinal tube and allowed to drip into a bottle standing in dry ice: this causes immediate freezing. When the juices are to be studied, they are thawed very slowly (in ice in the refrigerator) to prevent warming even for short periods. They are then quickly pipetted into a flask, pre-warmed to 56°C, and allowed to remain at that temperature for 30 minutes. Variation in temperature of $\pm$ 0.5°C is scrupulously avoided. Following this treatment they may be handled as any other biological fluid without danger of proteolysis of immunoglobins for long periods (at least a week, if kept reasonably cold).

2. Immunoelectrophesis of intestinal juice has been performed on many intestinal juice specimens. Distinct, reproducible patterns are now obtainable. This is shown in Figure (1). The heavy band in the gamma region is IgA. It was found in every intestinal juice so far examined, including juices before $(NH_4)_2 SO_4$ concentration of gamma globulins. The faint band more cathodal in position is IgG, and is found very infrequently in intestinal juice. When present, its intensity is always less than of IgA. The faint band anodal to IgA but cathodal to the origin is IgM. It is also very infrequently seen and typically is of low intensity when it does appear. The long band extending from the far reaches of the anodal region to past the origin into the cathodal region is found in every intestinal juice. The crosshatched markings near the origin indicate that occasionally this band oppears to be two, with intersect of non-identity as shown. This band is more granular a precipitate than that of IgA; is typically green-stained (bile), and is brought out by placing any normal goat serum or goat anti-human globulin serum into the trough. The nature of the serum or the intestinal juice component which goes into forming this precipitate is unknown. Reverse immunoelectrophoresis indicates, however, that the serum constituent is an alpha, not a gamma, globulin-therefore the precipitate is by definition not immunologic in nature.

Summarizing, intestinal juice analyzed by immunoelectrophoresis contains predominately IgA, very infrequently IgG and IgM, and a mysterious substance of greatly variable electrophoretic mobility which reacts with normal goat serum and all goatantisera.

3. Quantitation of immunoglobulins has been a difficult technical problem. The reason for this is that the use of standard specific antiserum-containing agar plates for quantitation (QP) gives a confusing picture of exactly which immunoglobulins are present in the secretions. Serum placed on a QP shows a single annular precipitate, the diameter of which is proportional to the concentration of the specific immunoglobulin in question. When intestinal secretions are placed on such a QP, several rings appear. The extra rings are due to the precipitate(s) described above between normal intestinal secretion and normal goat serum. It is sometimes very difficult to select from the two or three ring precipitates the one actually formed by immunoglobulin and antiserum. Therefore, immunoglobulin concentrations cannot accurately be determined without some means by which their precipitates can be identified.

A considerable effort has gone into solving this dilemma. The following procedures have been tried and found wanting for several reasons.

a. Normal serum has been placed in a well on the QP adjacent to the concentric precipitin rings seen with intestinal secretions. It was hoped that as the serum precipitin ring diffused toward the other rings, one of them (the actual immunoglobulin ring) would dissolve in antigen excess. This actually occurs, but it is very difficult to control, and this method has been abandoned for the moment.

b. QPs have been prepared from antiserum which has first been passed through DEAE—cellulose (phosphate buffer ph 7.4 M .025). Under these conditions only gamma globulins pass through the column while other proteins are retained. Since the extra precipitate seen in intestinal juice represents a reaction between the juice and alpha globulins in the serum, it was expected that they would not occur in QPs prepared from only the gamma proteins of antiserum. This was in fact, the case. The only problem with

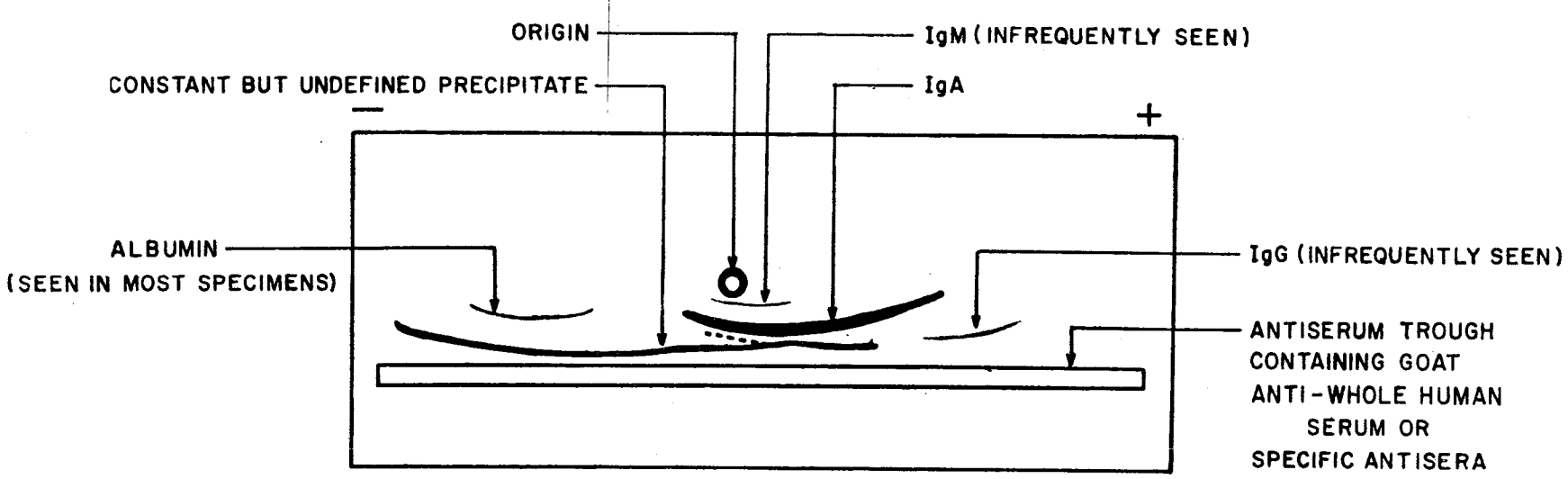

FIG. I. COMPOSITE DIAGRAMATIC REPRESENTATION OF AN IMMUNOELECTRO-PHORESIS OF CONCENTRATED NORMAL INTESTINAL JUICE

this method is that it is wasteful of antiserum, and also it is difficult to determine how much antiserum to mix with agar when preparing the plates. This latter problem arises with each batch of antisiserum passed through the column. Lastly, the method is very time consuming.

c. A third method would be to prepare two QPs, one with goat antiserum, and the other with normal goat serum (anti-nothing). The extra precipitate would appear on both, but only the QP with antiserum would show the immunoglobulin ring in addition. We have not tried this method, since the problems of standardizing the plates to insure that all give the same ring sizes (the extra precipitates) would be nearly impossible and very wasteful of reagents. All ring sizes would have to be the same, so that the actual immunoglobulin ring would be readily identified as unique.

The last method to be described is currently being refined, but seems to be accurate and easy to perform. In principal it involves adding serum to intestinal juice. with the expectation that the serum and intestinal juice immunoglobulins will combine to produce a single easily recognizable precipitin ring on a QP. A similar amount of serum in saline acts as a control, and the difference in ring sizes between the serum-intestinal juice mixture and the serum-saline mixture is taken as the contribution from the intestinal juice.

In detail the method is as follows: 0.1 cc of the unknown intestinal juice and 0.1 cc of saline (in duplicate) are added to test tubes. Then 0.1 cc of normal human serum in high dilution is added to each tube, and the contents mixed thoroughly. The mixture from each tube is placed on an IgG, IgA and IgM QP and the precipitin rings are measured in the standard way. Calculations are simply as follows:

$$\text{mgm\% immunoglobulin (A, G or M)} = (A\text{-}B) \times 2$$

where A = mgm% immunoglobulin in intestinal juice

B = mgm% immunoglobulin in saline

and the difference is doubled to account for the 1:2 dilution of intestinal juice occasioned by the addition of serum.

This method works because the precipitin ring produced by serum in intestinal juice is readily recognizable on a QP. It has been our practice not to consider significant a difference in ring diameter of 0.1 mm or less, since smaller differences are not easily read on QPs.

Insofar as minor difference in ring diameters are not easily read, it is important that the added serum be highly dilute. This insures that there will be small changes in mgm% as ring diameter changes, since the relationship of these two is logarithmic. To illustrate, our usual IgG standard curve shows that the difference between 3.6 and 3.8 mm ring size is 11 mgm%, and the difference between 5.6 and 5.8 is 44 mgm%. Although in both cases the difference is only 0.2 mm, the chance for error to be multipled is far greater if the ring is bigger. For these reasons separate standard curves at low concentrations of immunoglobulins (serum diluted with saline) have to be set up for this procedure.

The value of this method has yet to be proven by appropriate control procedures, but it has already been shown to be quite reproducible. Preliminary data indicate that IgA predominates in the intestinal juice of normal subjects, while IgG and IgM are frequently absent (Table 12). A similar table published in SMRL Annual Report for 1967 should be considered inaccurate; it was not based on this method.

Summary

Ongoing studies with intestinal juice samples obtained from normal human volunteers have met with some success amid considerable frustrations. A method has been developed to prevent proteolytic action against the immunoglobulins during study. This involves preliminary heating of juices at 56°C for 30 minutes. The immunoelectrophoretic pattern of these secretions has been analyzed and is reproducible Finally, one method from among many is under development to allow for quantitation of immunoglobulins in intestinal secretions. The quantities of IgA, IgM and IgG in the secretions of several normal patients are given, and confirm the predominance of IgA.

Table 12. Immunoglobulin content of intestinal fluids. Each subject was intubated on four occasions, hence four sets of data on each subject. Numbers to the left of vertical lines are from fluids as obtained; those to the right are on concentrated specimens of the same fluids.

	Immunoglobulin Concentration mgm/100 cc						Protein Concentration mgm/cc	
	IgG		IgA		IgM			
Subject 1	2.8	16.0	8.4	42.0	0	0	8.6	6.2
	2.8	8.2	8.4	31.1	0	0	7.8	4.9
	17.8	5.4	22.4	31.1	0	0	10.0	6.5
	4.8	8.2	14.8	48.7	0	0	9.6	4.5
Subject 2	4.8	19.2	7.6	23.7	3.2	5.6	11.3	12.6
	2.4	13.0	4.8	11.3	0	6.9	10.5	10.1
	0.6	13.0	1.6	11.3	0	6.9	13.0	9.1
	4.8	8.0	3.0	10.3	0	4.8	9.5	9.5
Subject 3	15.0	8.0	2.0	5.5	0	0	10.4	8.6
	6.8	6.8	3.0	14.8	0	0	13.0	12.4
	4.8	6.8	2.0	4.8	3.6	0	11.6	10.7
	4.8	16.0	3.8	24.9	3.6	0	15.2	10.4
Subject 4	2.8	14.8	8.4	28.0	0	0	9.0	4.4
	2.8	6.8	3.0	13.8	0	4.0	6.9	2.2
	2.8	9.8	9.6	44.2	0	6.0	9.3	6.7
	4.8	2.8	4.8	8.4	0	0	7.6	2.9
Subject 5	4.8	4.8	8.4	14.8	0	0	7.7	6.9
	4.8	0.8	3.0	3.0	0	0	6.8	5.1
	2.8	2.8	3.0	6.4	0	0	6.9	6.2
	2.8	0.8	2.0	8.4	0	0	6.8	6.9
Subject 6	4.8	2.8	1.0	0	0	0	7.2	2.2
	2.8	6.8	1.0	3.0	0	0	4.4	5.0
	2.8	4.8	1.0	6.4	0	0	4.7	3.9
	0	4.8	0	4.8	0	0	6.1	2.6
Subject 7	0	0	0	1.6	0	0	3.6	6.6
	0	0	0	1.6	0	0	5.3	2.2
	0	4.2	0	5.0	0	0	3.9	6.5
	0	0	0	2.2	0	0	2.2	2.4
Subject 8	0	0	1.2	8.6	0	0	7.7	4.4
	0	0	0	5.8	0	0	3.7	5.4
	0	0	4.0	15.0	0	0	6.3	5.0
	0	0	2.2	5.0	0	0	4.7	5.8
Subject 9	0	1.8	5.0	8.6	4.4	4.4	5.5	7.0
	1.8	0	5.0	12.6	7.2	7.2	7.7	7.0
	0	0	0	5.8	4.4	4.4	8.8	6.0
	0	1.8	2.2	17.2	7.2	7.2	8.9	11.0
Subject 10	9.8	9.8	12.6	90.0	7.2	13.0	4.9	5.2
	0	9.0	19.6	112.6	4.4	10.4	6.7	8.2
	1.8	4.2	38.4	225.6	10.4	21.0	7.2	8.6
	1.8	4.2	29.2	249.0	10.4	21.0	5.5	8.4
Subject 11	13.8	0.8	4.2	14.2	4.6	6.6	7.4	5.2
	8.0	3.2	13.0	45.6	6.6	7.4	4.0	4.0
	3.2	6.0	4.2	17.8	6.6	2.0	6.3	4.1
	3.2	3.2	3.4	20.2	4.6	4.6	9.5	4.0